

# Cadmium-induced early changes in $O_2^{\bullet-}$ , $H_2O_2$ and antioxidative enzymes in soybean (*Glycine max* L.) leaves

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**Abstract** Cadmium-induced initial changes in the production of reactive oxygen species (ROS) and antioxidant mechanism were investigated in soybean (*Glycine max* L. cv. Don Mario 4800 RR) leaves. Whole plants (WP) and plants without roots (PWR) were exposed to 0.0, 10.0 and 40.0  $\mu\text{M}$  Cd for 0, 4, 6 and 24 h. Compared to PWR, a higher level of endogenous Cd in WP was associated with a lower oxidative stress measured in terms of lipid peroxidation. Furthermore,  $O_2^{\bullet-}$  content decreased in the leaves of Cd-treated WP, whereas it increased in those of Cd-treated PWR. Although  $O_2^{\bullet-}$  accumulation in PWR was associated with a decrease in superoxide dismutase (SOD) activity,  $O_2^{\bullet-}$  diminution in WP leaves was not related to any increase in SOD activity.  $H_2O_2$  content increased in the leaves of both Cd-treated WP and PWR, and it was

concomitant with a corresponding decline in catalase (CAT) and ascorbate peroxidase (APX) activities. When diphenyl iodonium (DPI), an inhibitor of NADPH oxidase, was added,  $H_2O_2$  content remained unchanged in Cd-treated WP, suggesting that NADPH oxidase does not participate in the early hours of Cd toxicity. Taken together, our results showed that early ROS evolution and oxidative damage were different in WP and PWR. This suggests that the response in soybean leaves during the early hours of Cd toxicity is probably modulated by the root.

**Keywords** Antioxidant defense · Cd toxicity · NADPH oxidase · Oxidative stress · Reactive oxygen species · Soybean

## Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
DM	Dry mass
DPI	Diphenyl iodonium
FM	Fresh mass
LA	Leaf area
MDA	Malondialdehyde
NBT	Nitroblue tetrazolium
PVPP	Polyvinylpyrrolidone
PWR	Plants without roots
ROS	Reactive oxygen species
SOD	Superoxide dismutase
WP	Whole plants

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## Introduction

Heavy metals like cadmium (Cd) have been related to an overproduction of reactive oxygen species (ROS) that usually leads to an uncontrolled oxidative stress (Gallego et al. 1996; Dixit et al. 2001; Sandalio et al. 2001; Schützendübel and Polle 2002; Romero-Puertas et al. 2004). In that sense, long-term Cd-incubation (several weeks) of whole plants resulted in the accumulation of  $O_2^{\bullet-}$  and  $H_2O_2$ , and in lipid peroxidation (Gallego et al. 1996; Shah et al. 2001; Romero-Puertas et al. 2004); this effect has been characterized as a chronic oxidative damage (Romero-Puertas et al. 2004). On the other hand,  $O_2^{\bullet-}$  and  $H_2O_2$  increased in tobacco cell suspensions over short-term Cd-incubation (several min), and this has been related to an oxidative burst (Piqueras et al. 1999; Olmos et al. 2003). Moreover, different authors have suggested a role of  $H_2O_2$  in signaling Cd stress (Schützendübel et al. 2001; Romero-Puertas et al. 2004). Experiments with a specific inhibitor suggested that the main source of  $H_2O_2$  in plants under Cd stress could be the plasma membrane-bound NADPH oxidase complex (Olmos et al. 2003; Romero-Puertas et al. 2004; Horemans et al. 2007). However,  $O_2^{\bullet-}$  is the direct product of NADPH oxidase. Although it has been shown to act as a ROS signal under different stress conditions (Vranová et al. 2002), little is known about the mechanism involved in the generation of  $O_2^{\bullet-}$  and even less about its signaling activity in Cd-stressed plants.

The generation of ROS during the earliest hours of Cd toxicity has been observed in tobacco and *Arabidopsis* cell cultures (Olmos et al. 2003; Garnier et al. 2006; Horemans et al. 2007) and in roots of alfalfa seedlings (Ortega-Villasante et al. 2007). For instance,  $H_2O_2$  accumulation in either tobacco cell cultures or Scots pine roots seems to be caused by the induction of SOD activity and stabilization or inhibition of the system involved in  $H_2O_2$  removal (Olmos et al. 2003; Schützendübel et al. 2001).

To our knowledge, the evolution of ROS in the leaves of Cd-treated whole plants (WP) has not been studied. Therefore, the objective of this study was to examine the levels of  $O_2^{\bullet-}$  and  $H_2O_2$  vis-à-vis the evolution of antioxidant enzymes involved in their degradation during the early stages of Cd toxicity. As roots contain very high concentrations of Cd

(Sandalio et al. 2001; Shah et al. 2001) and is a potential source of signals to the shoot (Capone et al. 2004; Hsu and Kao 2005), the evolution of ROS in leaves was also assessed in an experimental system of Cd-treated plants without roots (PWR).

## Materials and methods

### Plant material and growth conditions

Soybean (*Glycine max* cv. Don Mario 4800 RR) seeds were germinated on Whatman filter paper soaked with distilled water in a growth chamber at 25°C in the dark for three days. Seedlings were then provided with a photosynthetic photon flux density at 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  using a combination of fluorescent tubes and tungsten lamps under a 16 h photoperiod. They were grown in aerated Hoagland nutritive solution for 12 days until the first pair of leaves was fully expanded. Since Cd uptake is improved at pH 5.0 (Schützendübel et al. 2001), the Hoagland nutrient solution was adjusted to this pH in order to acclimatize plants for Cd treatment. Whole plants (WP) and plants without roots (PWR) were transferred to 0.0, 10.0, 40.0 and 80.0  $\mu\text{M}$   $\text{CdCl}_2$  in Hoagland nutritive solution (pH 5.0) and kept under the same light and temperature conditions, as described above. Leaf samples were collected at 0, 4, 6 and 24 h after initiating the Cd stress. Cd toxicity was evaluated by measuring growth parameters, such as fresh leaf mass (FM), leaf area (LA), dry leaf mass (DM) and root length. The latter was measured according to Schützendübel et al. (2001), with minor modifications. Root length measurements were taken by labelling roots 5 mm below the tip with water-resistant ink immediately before Cd treatment. The distance between the tip and ink label was then measured after 4, 6 and 24 h of Cd treatment.

### Estimation of Cd content

Five roots and ten leaves were dried at 80°C at constant weight and then digested in  $\text{HNO}_3$ . The concentration of Cd in roots was determined by acetylene-air-flame mode atomic absorption spectrophotometry (Varian Spect AA, model 50; Varian, Inc., Palo Alto, USA), with a Cd hollow-cathode

lamp set at a detection wavelength of 228.8 nm using Cd Tritisol standard solution (Merck KGaA, Darmstadt, Germany). The content of Cd in leaves was measured by inductively coupled plasma optical emission spectrometry (ICP-OES) using a spectrometer (model ICP-2070; Baird, Bedford, USA). Cd emission signal was monitored at 228.802 nm. Cd standard solutions were prepared by appropriate dilutions of a 1000 mg l<sup>-1</sup> stock solution using 0.1 M HNO<sub>3</sub> as a diluent.

#### O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> measurements

Superoxide anion content was measured spectrophotometrically, according to Chaitanya and Naithani (1994). About 200 mg fresh samples were homogenized in cold 100 mM sodium phosphate buffer (pH 7.2) containing 1 mM diethyl dithiocarbamate to inhibit SOD activity. Superoxide anion content was determined in the supernatant by its ability to reduce NBT. The assay mixture contained 100 mM sodium phosphate buffer (pH 7.2), 1.0 mM diethyl dithiocarbamate, 0.25 mM NBT plus the supernatant in a final volume of 3 ml. Unspecific NBT reduction was discarded by subtracting the absorbance at 540 nm of samples previously treated with bovine SOD from the absorbance of samples treated with diethyl dithiocarbamate. The kinetics of NBT reduction was followed during 30 s at 540 nm in a Perkin Elmer DU70 (Perkin Elmer, Inc, Waltham, USA) spectrophotometer. Superoxide anion content was expressed as ΔA 540 nm min<sup>-1</sup> mg<sup>-1</sup> FW. The specificity and accuracy of O<sub>2</sub><sup>•-</sup> determination of this spectrophotometric method were confirmed as follows: (a) NBT reduction significantly decreased in the absence of diethyl dithiocarbamate (SOD inhibitor), or with bovine SOD previously added to the sample extracts (data not shown); and (b) although Cd is not a redox-active metal, a possible interference of CdCl<sub>2</sub> on NBT reduction was ruled out because increasing concentrations of CdCl<sub>2</sub> (0, 10, 40 and 80 μM) did not inhibit NBT reduction in control reaction mixtures (without extract; data not shown) in vitro.

The content of H<sub>2</sub>O<sub>2</sub> in leaves was determined by a spectrophotofluorometric method based on Guilbault et al. (1968). Briefly, 200 mg of material (FM) was homogenized in 1.5 ml 30 mM phosphate buffer (pH 7.4) and 120 mM NaCl in an ice-cold mortar. The homogenate was centrifuged at 15,000g for

20 min. An aliquot of the supernatant was incubated in a solution containing 100 mM phosphate buffer (pH 7.4), 2.8 U ml<sup>-1</sup> of horseradish peroxidase (Boehringer, Mannheim, Germany) and 400 μM *p*-hydroxyphenylacetic acid. The fluorescence of the oxidized product (excitation light, 317 nm; emission light, 414 nm) was recorded with a spectrofluorometer (model RS1501; Shimadzu, Kyoto, Japan). In order to discount unspecific oxidation of *p*-hydroxyphenylacetic acid mediated by substances other than H<sub>2</sub>O<sub>2</sub>, catalase (Sigma, Missouri, USA) was added to the extract 30 min before the addition of horseradish peroxidase. Fluorescence was transformed into molar H<sub>2</sub>O<sub>2</sub> concentration using a linear calibration curve (0–1 μM), and H<sub>2</sub>O<sub>2</sub> content was expressed as nmol H<sub>2</sub>O<sub>2</sub> mg<sup>-1</sup> FM.

#### In-vivo NADPH oxidase activity

In-vivo NADPH oxidase activity was indirectly assessed by the ability of diphenyl iodonium (DPI) to inhibit it. In-vivo inhibition of NADPH oxidase was performed by adding 5 μM DPI (Jiang and Zhang 2002) to the nutritive solution supplemented with 0, 10 or 40 μM Cd. Leaf samples for this assay were taken after 24 h of treatment, and H<sub>2</sub>O<sub>2</sub> content was determined as described above.

#### Antioxidative enzyme assay

Enzyme extractions were carried out at 4°C. Frozen plant tissues were ground to a fine powder in liquid nitrogen using a pestle and mortar and extracted at a ratio of 1:3 w/v fresh mass in extraction buffer (100 mM potassium phosphate buffer [pH 7.4] containing 1 mM EDTA and 1% w/v insoluble PVPP). The homogenate was centrifuged at 33,000 × g for 30 min at 4°C, and the supernatant was used for the antioxidant enzyme analyses.

Total SOD activity was assayed at 560 nm by measuring the inhibition of the photochemical reduction of NBT, according to Beauchamp and Fridovich (1973). One unit of SOD activity was defined as the amount of enzyme producing a 50% inhibition of the photochemical reduction of NBT. SOD activity was expressed as SOD units mg<sup>-1</sup> protein.

Total APX activity was measured by the decrease in absorbance of ascorbate at 290 nm according to Nakano and Asada (1981). The reaction mixture

contained appropriate dilutions of the samples in 50 mM phosphate buffer (pH 7.4), 0.5 mM ascorbic acid and 0.1 mM H<sub>2</sub>O<sub>2</sub>. APX activity was expressed as nmol ascorbate min<sup>-1</sup> mg<sup>-1</sup> protein.

CAT activity was measured spectrophotometrically by the disappearance of H<sub>2</sub>O<sub>2</sub> at 240 nm according to Aebi (1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), the sample and 10 mM H<sub>2</sub>O<sub>2</sub>. The assay was performed at 25°C in a 1 ml cuvette, and CAT activity was expressed as nmol H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> mg<sup>-1</sup> protein.

### Biochemical assays

Proteins were determined according to Bradford (1976), using bovine serum albumin as a calibration standard. Lipid peroxidation was evaluated as MDA content according to Heath and Packer (1968). Additionally, a control value without thiobarbituric acid was determined as per Hodges et al. (1999). MDA content was calculated by using an extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup>, and results were expressed as nmol MDA g<sup>-1</sup> FW.

### Statistical analyses

In general, data were averaged over three independent experiments; each experiment was based on at least two measurements of pooled material from each treatment. Data were analyzed by one-way ANOVA followed by the comparison of mean values by Fisher's least significant difference (LSD) test, using InfoStat 2005 (InfoStat/Professional, Version 2005 p.1; Grupo InfoStat/FCA, Universidad Nacional de Córdoba, Córdoba, Argentina). The term significant has been used to indicate differences for which  $P \leq 0.05$ .

## Results

### Effect of Cd on seedling growth

Preliminary experiments were carried out to determine the range of Cd concentrations which soybean seedlings could be subjected to without developing visual symptoms of Cd toxicity. Cd treatment did not induce chlorosis in leaves throughout the experiment. However, Cd at 80 µM significantly inhibited the

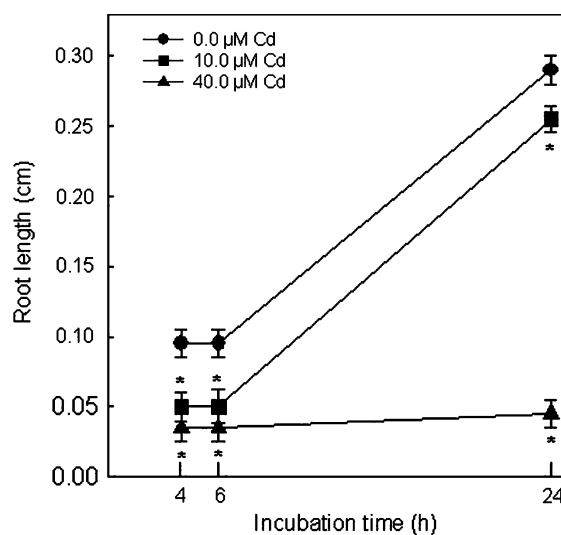
growth of roots and the first pair of trifoliate leaves (data not shown). Cd at lower concentrations had different effects on root and shoot growth; root growth was delayed at 10 µM, but completely inhibited at 40 µM Cd (Fig. 1). Although neither of these two concentrations affected leaf growth measured as fresh mass (Table 1), Cd at 40 µM significantly decreased leaf area and dry mass (Table 1). Based on these results, Cd at 10 and 40 µM was used in subsequent experiments.

### Tissue Cd content in WP and PWR

The accumulation of Cd in soybean seedlings was greater in roots (ppm) than in leaves (ppb) (Table 2). Despite the high precision of ICP-OES, Cd content in the leaves of WP and PWR subjected to 10 µM Cd was lower than the equipment's detection limit (10 µg l<sup>-1</sup>) throughout the entire experiment. This was also observed in leaves during the first hours of treatment with 40 µM Cd. However, Cd could be determined accurately in leaves subjected to 40 µM Cd after 24 h (Table 2). These results showed that Cd content was higher in WP than in PWR leaves.

### ROS evolution in WP and PWR

The results showed that the evolution of ROS in the leaves of WP and PWR was different. Although Cd at



**Fig. 1** Effect of Cd stress on the root growth of soybean seedlings. \* Significantly different from the control at  $P \leq 0.05$ , according to least significant difference (LSD) test

**Table 1** Effect of Cd stress on leaf area, fresh mass and dry mass of soybean seedlings exposed to CdCl<sub>2</sub> for 24 h at the whole plant (WP) level

Cd (μM)	Leaf area (cm <sup>2</sup> )	Fresh mass (g)	Dry mass (g)
0.0	16.5 a <sup>a</sup>	2.9 a	0.210 a
10.0	17.4 a	2.9 a	0.228 a
40.0	13.0 b	2.8 a	0.181 b

<sup>a</sup> Averaged over two replications from three independent experiments; means with common letters are not significantly different at  $P \leq 0.05$ , according to least significant difference (LSD) test

**Table 2** Cd content (mg g<sup>-1</sup> dry mass)<sup>a</sup> in roots and leaves of soybean seedlings subjected to 40.0 μM Cd for 24 h

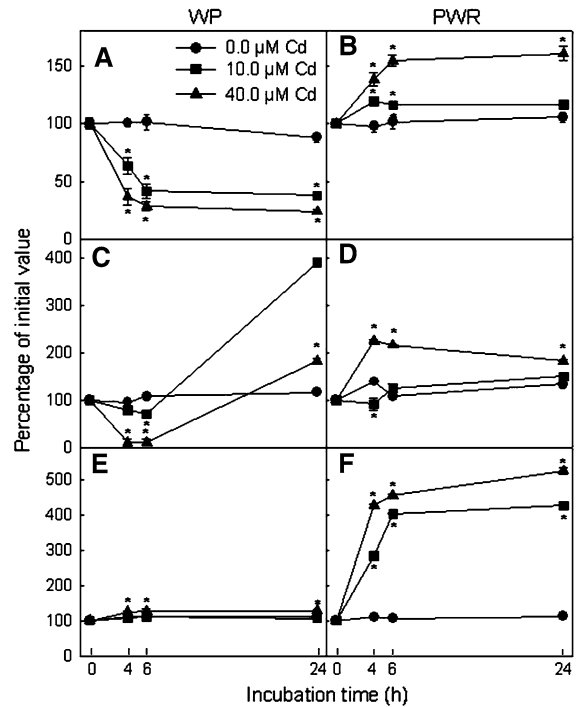
Plant without root	Whole plant	
Leaf (ppb)	Leaf (ppb)	Root (ppm)
0.0015	0.0033	1.87

<sup>a</sup> Averaged over two independent experiments

both concentrations decreased the content of O<sub>2</sub><sup>•-</sup> in WP leaves, it could increase the level of O<sub>2</sub><sup>•-</sup> in PWR leaves only at 40 μM (Fig. 2a–b). In all cases, the level of O<sub>2</sub><sup>•-</sup> in leaves after 24 h was similar to that determined after 6 h of Cd treatment (Fig. 2a–b). In WP leaves, Cd at both concentrations caused a decrease in H<sub>2</sub>O<sub>2</sub> content; however, in PWR leaves, an increase in H<sub>2</sub>O<sub>2</sub> occurred during the first hours of treatment with 40 μM, but not with 10 μM Cd (Fig. 2c–d). After 24 h of treatment, H<sub>2</sub>O<sub>2</sub> content increased in WP leaves, while the level of H<sub>2</sub>O<sub>2</sub> in PWR leaves was already stable after 6 h of Cd treatment (Fig. 2c–d).

#### Oxidative damage in WP and PWR

Although MDA increased in both WP and PWR, the results showed that the rate of MDA accumulation was markedly different between the two experimental systems. In WP leaves, MDA content increased by 30 % when treated with 40 μM Cd (Fig. 2e). On the other hand, in PWR leaves, Cd at both concentrations increased MDA content by about 450%, as compared to controls (Fig. 2f). In all these leaves, the level of MDA content during the first 6 h of Cd treatment was comparable to that attained after 24 h in the control.



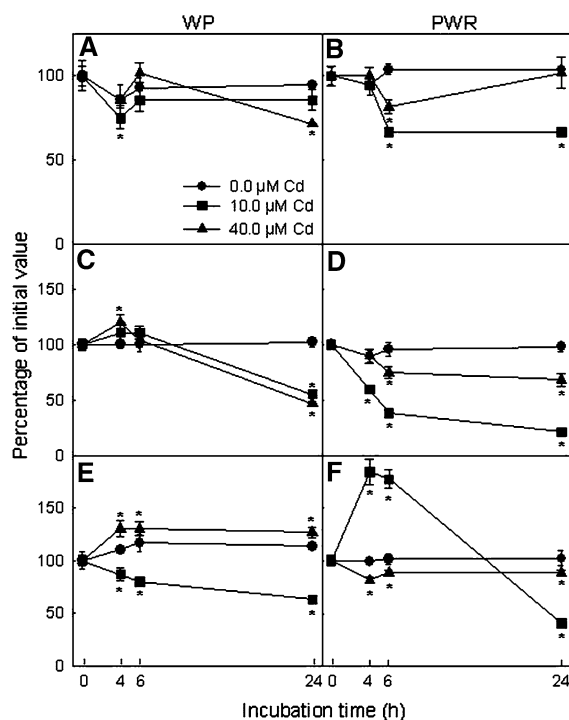
**Fig. 2** Effect of Cd stress on O<sub>2</sub><sup>•-</sup> (a, b), H<sub>2</sub>O<sub>2</sub> (c, d) and MDA (e, f) contents in soybean leaves. The contents of O<sub>2</sub><sup>•-</sup>, H<sub>2</sub>O<sub>2</sub> and MDA are expressed as a percentage of the initial value (100% = value of each parameter before the initiation of Cd stress). Mean initial values are: O<sub>2</sub><sup>•-</sup> = 0.023 Δ A 540 nm min<sup>-1</sup> mg<sup>-1</sup> fresh mass; H<sub>2</sub>O<sub>2</sub> = 0.172 nmol - H<sub>2</sub>O<sub>2</sub> mg<sup>-1</sup> fresh mass; MDA = 0.011 nmol MDA mg<sup>-1</sup> FW. \* Significantly different from the control at  $P \leq 0.05$ , according to least significant difference (LSD) test. PWR, plants without roots; WP, whole plants

#### Antioxidant enzymes in WP and PWR

Total SOD activity remained mainly unchanged in WP leaves, but had a tendency to decrease in the leaves of PWR, especially at 10 μM Cd (Fig. 3a–b). The other assayed antioxidant enzymes, such as CAT and APX exhibited a tendency to decrease with increasing levels of Cd in both experimental systems (Fig. 3c–f).

#### In-vivo effect of Cd on NADPH oxidase

In order to investigate the source of O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> generated in plants under Cd stress, in-vivo NADPH oxidase activity was studied. NADPH oxidase catalyzes the production of O<sub>2</sub><sup>•-</sup>, and is involved in subsequent generation of H<sub>2</sub>O<sub>2</sub> by an extracellular SOD. DPI, an inhibitor of NADPH oxidase, severely

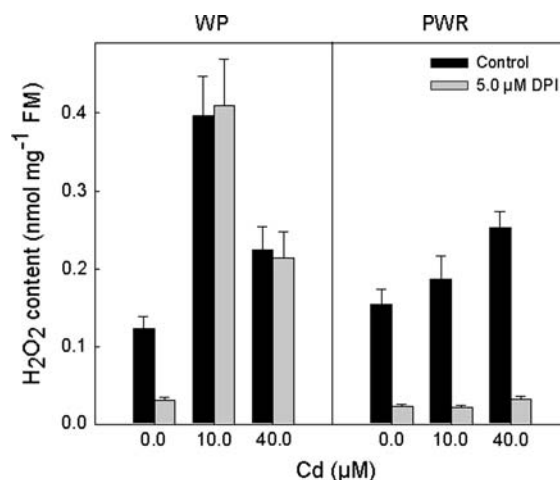


**Fig. 3** Effect of Cd stress on SOD (a, b), CAT (c, d) and APX (e, f) activities in soybean leaves. SOD, CAT and APX activities are expressed as a percentage of the initial value (100% = value of each parameter before the initiation of Cd stress). Mean initial values are: SOD, 2.8 U mg<sup>-1</sup> protein; CAT, 0.413 nmol H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> mg<sup>-1</sup> protein; APX, 2.68 nmol ascorbate min<sup>-1</sup> mg<sup>-1</sup> protein. \* Significantly different from the control at  $P \leq 0.05$ , according to least significant difference (LSD) test. APX, ascorbate peroxidase; CAT, catalase; PWR, plants without roots; SOD, superoxide dismutase; WP, whole plants

decreased H<sub>2</sub>O<sub>2</sub> content in the leaves of PWR and WP under control conditions (Fig. 4), suggesting that this inhibitor was taken up and transported to leaves. On the other hand, when PWR were simultaneously fed with Cd and DPI there was a marked decline in H<sub>2</sub>O<sub>2</sub> content in leaves, as compared to that in the leaves of Cd-treated plants. In contrast, feeding Cd and DPI to WP did not affect the content of leaf H<sub>2</sub>O<sub>2</sub>, suggesting that NADPH oxidase is not involved in its generation (Fig. 4).

## Discussion

The present study based on WP and PWR experimental systems in soybean seedlings suggested that roots might exert an influence on Cd-mediated



**Fig. 4** Regulation of leaf H<sub>2</sub>O<sub>2</sub> content by diphenyl iodonium (DPI) in leaves of soybean WP (a) and PWR (b) under Cd stress for 24 h. DPI was simultaneously added with Cd in the nutrient solution. Mean initial value: H<sub>2</sub>O<sub>2</sub>, 0.193 nmol mg<sup>-1</sup> fresh mass. PWR, plants without roots; WP, whole plants

oxidative stress in leaves. It has previously been shown that a high endogenous Cd level is associated with an increased oxidative stress in plants (Dixit et al. 2001; Sandalio et al. 2001; Shah et al. 2001; Pereira et al. 2002). However, in this study, WP with a high endogenous leaf-Cd content exhibited less oxidative stress than PWR, as evidenced by the levels of MDA (lipid peroxidation). These results show that oxidative damage in WP is not related to metal concentrations during the first hours of Cd toxicity. Other events related to plant defences against Cd toxicity could be taking place. For instance, the production of phytochelatins has been reported (Heiss et al. 2003), although they would not be the first mechanism linked to heavy metal tolerance (Lee et al. 2003). Instead, Ortega-Villasante et al. (2007) suggested that ROS signalling networks might be involved in the early stages of a homeostatic response to Cd toxicity.

In this study, the content of O<sub>2</sub><sup>•-</sup> decreased in WP during the first hours of Cd treatment. This is in contrast with the promoting effect of Cd on O<sub>2</sub><sup>•-</sup> content in PWR leaves as obtained in this study and also with the results reported by Olmos et al. (2003) in tobacco cell suspensions. Furthermore, to our knowledge, this is the first report of a decrease in O<sub>2</sub><sup>•-</sup> content in the leaves of Cd-treated plants. However, it is not the first evidence for a down-regulation of O<sub>2</sub><sup>•-</sup> level by abiotic stresses



(Rodriguez et al. 2004). It can be argued that this decrease in  $O_2^{\bullet-}$  content in WP leaves could be due to an enhanced SOD activity. However, total SOD activity remained unchanged in WP leaves during the early hours of Cd treatment. Nevertheless, we cannot rule out the possibility that  $O_2^{\bullet-}$  dismutation may occur by an enhanced activity of a specific SOD isozyme. By contrast, results in PWR show a close relation between an increase in  $O_2^{\bullet-}$  content and a decrease in SOD activity.

Some investigations have shown that  $H_2O_2$  increases in cells under Cd stress (Piqueras et al. 1999; Schützendübel et al. 2001; Schützendübel and Polle 2002; Olmos et al. 2003). Our results are in agreement with these previous observations because  $H_2O_2$  increased in the leaves of WP and PWR treated with Cd for 6 h or more. The accumulation of  $H_2O_2$  in soybean leaves was correlated to a lack of response of CAT and APX activities. In this context, Schützendübel et al. (2001) and Olmos et al. (2003) reported that Cd inhibited the enzymes involved in  $H_2O_2$  removal. Moreover, when soybean leaf extracts were incubated with 10 or 40  $\mu M$  Cd a decrease in CAT activity occurred (data not shown), suggesting a direct inhibition of this enzyme. It has been proposed that the mechanism by which Cd induces a decrease in antioxidative defense is mostly related to its ability of being sequestered by thiol-containing biomolecules (Grill et al. 1991).

Interestingly, a higher level of  $H_2O_2$  in WP than in PWR was not associated with higher oxidative damage. Thus, in PWR leaves exposed to 10  $\mu M$  Cd a 450% increase in MDA level was accompanied by a 0.3-fold increase of  $H_2O_2$ , whereas in WP leaves a 30% increase in MDA level was associated with a 4.5-fold increase of  $H_2O_2$ . This low MDA/ $H_2O_2$  ratio could be suggesting that ROS have a signaling role in the leaves of Cd-treated WP. Accordingly, many reports have identified  $H_2O_2$  as a signal molecule in response to Cd stress (Schützendübel et al. 2001; Romero-Puertas et al. 2004; Ortega-Villasante et al. 2007). It has been suggested that  $H_2O_2$  production in Cd-treated cells could be a regulated event with calmodulin and Ca-dependent protein kinase (CDPK) implicated in its signal transduction sequence (Olmos et al. 2003).

Since NADPH oxidase might be involved in the generation of ROS during the oxidative burst in Cd-treated plant cells (Piqueras et al. 1999; Olmos et al. 2003), we studied its association with the production of ROS in both soybean experimental systems.

Modifications of  $H_2O_2$  accumulation by DPI showed that the NADPH oxidase complex was active in WP and PWR leaves under control conditions. By contrast, the lack of DPI-effect on  $H_2O_2$  content of Cd-treated WP suggested that NADPH oxidase was not involved in the generation of ROS in their leaves. Although an interference by Cd in preventing DPI uptake cannot be ruled out, the observed decrease in  $O_2^{\bullet-}$  content in WP leaves, which cannot be ascribed to SOD activity, could be attributed to the down-regulation of NADPH oxidase.

On the contrary, the increase in  $O_2^{\bullet-}$  content together with an inhibition of  $H_2O_2$  level by 5.0  $\mu M$  DPI in Cd-treated PWR leaves strongly suggests that NADPH oxidase was active. Similar results were observed in Cd-treated cell cultures (Piqueras et al. 1999; Olmos et al. 2003; Garnier et al. 2006; Horemans et al. 2007). The lack of NADPH oxidase activity in response to Cd stress in WP, as observed in this study, is in sharp contrast with its reported induction by either biotic or Cd stresses in plants cells. Further investigation is needed to determine whether this phenomenon is common to the early stages of heavy metal stress in plants.

Taken together, our results show that soybean leaves respond differently to Cd toxicity depending on the presence of root. As oxidative damage was more extensive in Cd-treated PWR than in WP, it is possible that roots may sense Cd in the environment and subsequently transmit the appropriate signal to the shoot. Supporting the idea of root signaling in plants under Cd stress, some investigations have suggested  $H_2O_2$  (Capone et al. 2004), salicylic acid (Alvarez 2000; Metwally et al. 2003), nitric oxide (Lamattina et al. 2003) and abscisic acid (Hsu and Kao 2003, 2005) as possible signals involved in the alleviation of Cd-induced toxic effects.

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